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A Novel Phenolic Glycolipid from *Mycobacterium leprae* Possibly Involved in Immunogenicity and Pathogenicity

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A phenolic glycolipid was obtained in high amounts (2% of dry weight) from *Mycobacterium leprae* isolated from infected armadillo liver. Infrared and nuclear magnetic resonance spectroscopy showed that it is closely related to "mycoside A" from *Mycobacterium kansasii* and is therefore a glycosylphenolic phthiocerol diester. The crucial difference between the two products is in the composition of the attached trisaccharide. Gas-liquid chromatography-mass spectroscopy showed that the product from *M. kansasii* is composed of 2,4-di-*O*-methylrhamnose, 2-*O*-methylrhamnose, and 2-*O*-methylfucose, whereas that from *M. leprae* contains 2,3-di-*O*-methylrhamnose, 3-*O*-methylrhamnose, and 3,6-di-*O*-methylglucose. The distinct composition of the oligosaccharide segment of the glycolipid from *M. leprae* may make it useful for the chemical and serological differentiation of this organism from other mycobacteria. Surprisingly large quantities (2.2 mg/g of dry liver) of the glycolipid were also found in infected liver residue freed of *M. leprae*, suggesting that it may be responsible for the electron-transparent "foam" surrounding the organism in infected lepromatous tissue.

Until recently, research in leprosy has been impeded by lack of adequate supplies of the etiological agent, *Mycobacterium leprae*, because the organism is extremely fastidious and incapable of growth in vitro. The only sources of *M. leprae* previously available were from infected human tissue and mouse foot pad tissue. The recent availability of large amounts of *M. leprae*-infected armadillo tissue provides an opportunity for expanded research. We are interested in the mechanism of pathogenesis of *M. leprae* and in its antigenic composition. In particular, we seek species-specific antigens which could be used for the development of diagnostic skin tests and serological tests and in exploring the disordered cellular immunity so characteristic of some forms of leprosy.

Leprosy is the most serious of a variety of diseases caused by nontuberculous (atypical) mycobacteria. Recently, we characterized the specific surface antigens of a host of atypical mycobacteria as "C-mycosidic" glycopeptidolipids, composed of an invariant monoglycosylpeptidolipid "core" and a variable species- or type-specific tri- or tetrasaccharide (6, 7; P. J. Brennan, Rev. Infect. Dis., in press). Moreover, the C-mycosidic antigens comprise the bulk of the superficial cell wall sheath which surrounds certain atypical mycobacteria and which apparently shields them within phagolysosomes from lysosomal enzymes (3). This information prompted us to seek like substances and roles in *M. leprae*. Indeed, we have reported that an impure apolar lipid fraction from *M. leprae* yielded distinct lines of precipitation with antisera from lepromatous patients and from infected armadillos and did not react with antisera from patients with other mycobacterial infections (5). Characteristics of the lipid fraction indicated that the active substance might be related to the aromatic glycolipids of mycobacteria, the so-called "mycosides A," "B," and "G." These were previously isolated from *Mycobacterium kansasii* (12), *Mycobacterium bovis* (9), and *Mycobacterium marinum* (21), respectively. These are glycosides of ρ-phenol with a branched glycolic chain, the hydroxyl functions of which are esterified with normal and branched-chain fatty acids (13). They differ by the nature and number of sugar molecules which are linked to the phenolic hydroxyl group of the aglycone. We now report on the isolation from the serologically active lipid preparation derived from *M. leprae* of a major glycolipid which conforms to the specificiations of a phenolic mycoside, but in which the unique carbohydrate composition suggests the role of a species-specific antigen. Moreover, the presence of high amounts of the glycolipid in the tissue surrounding the bacilli implies a function in the mechanism of the disease process. A brief abstract of this work has appeared (S. W. Hunter and P. J. Brennan,

72nd Amer. Soc. Biol. Chem. Annu. Meet., St. Louis, Mo., abstr. 34, 1981).

MATERIALS AND METHODS

Isolation of *M. leprae* from infected armadillo livers. Infected livers were obtained from W. F. Kirchheimer, U.S. Public Health Service Hospital, Carville, La., through D. D. Gwinn, Leprosy Program Officer, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The armadillos used had been infected with either 10^3 or 10^5 bacilli and sacrificed 1 to 3 years later (18, 25). The number of acid-fast bacilli (AFB) per gram of liver ranged between 1×10^9 and 9×10^{10}. Irradiated (2.5 Mrad) livers (10 to 25 g) were homogenized in a solution containing 0.135 M NaCl, 0.2 M Tris, 0.01 M $MgSO_4$, and centrifuged at $10,000 \times g$ for 10 min. The supernatant fluid, which was devoid of AFB, was retained and examined separately for the presence of the specific glycolipid. *M. leprae* was isolated from the $10,000 \times g$ pellet by the gentle method of Draper (10). Recovery of bacilli was variable, e.g., 0.09% (dry weight of bacilli/wet weight of liver) from a liver with an AFB titer of 1.76×10^9, compared with 0.9% from a liver with an AFB titer of 9.61×10^{10}.

Fractionation of lipids from *M. leprae* and from infected liver. *M. leprae* (50 to 100 mg, dry) was extracted twice with 10 ml of $CHCl_3$-CH_3OH (2:1) at 50°C for 18 h. The dried lipids were applied to a column (0.5 by 10 cm) of silicic acid (Mallinckrodt)-Celite (Johns-Manville) (2:1) and eluted with $CHCl_3$ (10 ml) followed by 2% CH_3OH in $CHCl_3$. Infected homogenized liver (10 g) was extracted by the Bligh-Dyer monophasic extractant (4). The dried lipid extracts were triturated with distilled ether, and the ether-soluble lipids were separated on silicic acid as described above. Likewise, lipids from lyophilized $10,000 \times g$ supernatant fluid (*M. leprae* free) from homogenized infected liver were fractionated by differential solubility in ether and by silicic acid column chromatography. In each case, the lipids in the 2% CH_3OH in $CHCl_3$ eluates were dissolved in hexane-ether (1:1) and applied to other silicic acid columns which were developed with hexane-ether (1:1), hexane-ether (1:3), and ether. The latter fraction contained the highly purified glycolipid. Removal of a slight impurity was possible on account of its insolubility in hexane (boiling point, 69°C).

Isolation of phenolic glycolipid (mycoside A) from *M. kansasii*. *M. kansasii* grown on Middlebrook's 7H11 medium was extracted with $CHCl_3$-CH_3OH (2:1) as described previously (6). The lipid (3 g) from 21 g of dry cells was applied to a column (2.5 by 41 cm) of DEAE-cellulose (acetate) (6) in $CHCl_3$ and eluted with 300 ml of $CHCl_3$ followed by 300 ml of 10% CH_3OH in $CHCl_3$. Lipid from the mixed eluates (1.7 g) was triturated with ether and the soluble lipids (1 g) in hexane-ether (1:1) were applied to a column of silicic acid-Celite (2.5 by 8 cm) which was irrigated with 100 ml each of hexane-ether (1:1), hexane-ether (1:3), and ether. The ether eluates contained only one lipid (R_f of 0.4 in ether-acetone, 9:1) which showed an infrared spectrum identical to that which has been published for mycoside A (12). About 60 mg of the pure lipid was recovered after these steps.

Analytical procedures. Thin-layer chromatography was conducted on commercial silica gel G plates from Fisher Corp., St. Louis, Mo. Glycolipids were located with orcinol-H_2SO_4 as described previously (8). For analysis of the sugar composition, glycolipids (1 mg) were hydrolyzed with 2 M trifluoroacetic acid and converted to alditol acetates by reduction with sodium borohydride followed by acetylation with acetic anhydride (1). Gas-liquid chromatography (GLC) of alditol acetates was conducted on a column (180 by 0.2 cm) of 3% SP-2340 on 100- to 120-mesh Supelcoport (Supelco, Inc.) as described previously (7). GLC-mass spectroscopy of alditol acetates employed the same column and a Finnigan quadrupol system (model 3200 E) coupled to a model 6000 data and graphic output system. Structural assignments were partly based on a comparison of fragmentation patterns with those of Jansson et al. (16) and Brennan et al. (6, 7). UV absorption was measured with a Perkin-Elmer Coleman 124 double-beam spectrophotometer at a concentration of 4 to 6 mg/ml of ethanol. Infrared absorption was determined with a Beckman IR-4240 spectrophotometer at a concentration of 25 mg/ml of CCl_4. Nuclear magnetic resonance (NMR) spectroscopy was conducted at a concentration of 13.2 mg of glycolipid per ml of $CDCl_3$ on an NT-360 spectrometer. To identify anomeric proton signals, glycolipids were first treated with 10% KOH in benzene-methanol (1:2; refluxing for 16 h) to destroy ester bonds, followed by 1 N methanolic-hydrochloric acid (refluxing for 5 h) to cleave glycosidic links. The NMR spectra of the degraded lipids were then compared with those of the native lipid.

The sources or synthesis of the authentic *O*-methyl sugars used for comparative purposes in this work have been described previously (6, 7). *O*-Methyl sugars were demethylated by the highly sensitive procedure described before (7).

RESULTS

Structure of the major lipid in the serologically active lipid preparation from *M. leprae*. Previously, we reported that serologically active lipid fractions from *M. leprae* contained one major glycolipid component (5). In the present work, this glycolipid, largely free of other lipids, was obtained by developing a silicic acid column with 2% CH_3OH in $CHCl_3$ after first charging the column with $CHCl_3$. The lipid was purified by further column chromatography, using hexane-ether mixtures. It was compared with the pure mycoside A obtained from *M. kansasii* in like fashion. Both lipids appeared as white solids readily soluble in such apolar solvents as hexane and ether. On thin-layer plates (Fig. 1) both gave an identical red-brown color response to orcinol. However, they had markedly different mobilities. Infrared spectra of the two (Fig. 2) were almost identical, characterized by the absorption bands of an aromatic nucleus (1,510 and 1,605 cm^{-1}), ester absorption at 1,725 cm^{-1}, and hydroxyl group absorption at 3,400 cm^{-1}. The

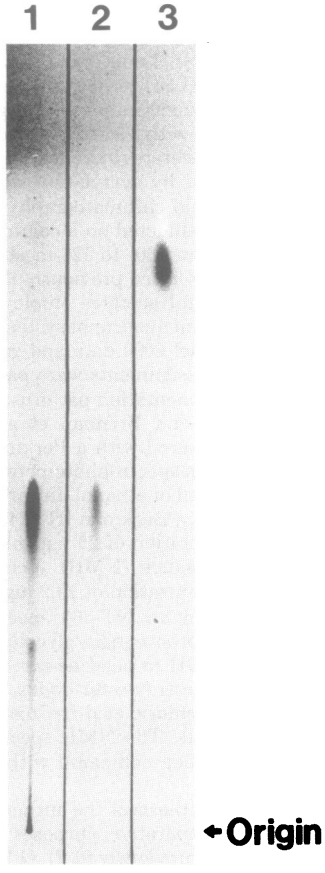

FIG. 1. *Thin-Layer chromatography of the phenolic glycolipids from M. leprae and M. kansasii. (1) The 2% CH_3OH-$CHCl_3$ eluate of the total M. leprae lipid fraction from silicic acid column. (2) The pure M. leprae phenolic glycolipid. (3) The pure M. kansasii glycolipid. Plate was chromatographed in ether-acetone (8:2), sprayed with 0.1% orcinol in 40% H_2SO_4, and heated.*

UV absorption spectra of the pure lipids from both sources were also identical, with two very strong peaks at 228 and 274 nm (with a shoulder at 280 nm). A bathochromic shift and increase in absorption was observed upon addition of 1 N NaOH to ethanolic solutions of both glycolipids, again indicating the presence of a phenolic group.

The NMR spectra of the phenolic glycolipids from *M. leprae* and *M. kansasii* were also compared (Fig. 3). Both contained two doublets in the low-field region 6.92 to 7.14 ppm characteristic of a phenolic nucleus with its *para* position linked to an aliphatic chain. A comparison of the singlets and doublets in the range 3.30 to 3.70 ppm indicated that the glycolipids from *M. leprae* and *M. kansasii* differ in their content of $-OCH_3$ groups. The singlet at 2.17 ppm from the *M. kansasii* product pointed to the presence of an acetyl substituent, probably attached to a sugar moiety as in the case of the C-mycoside antigens (3). The absence of such a signal in the *M. leprae* lipid presumably indicates the lack of sugar *O*-acetyl groups. There are other features of similarity and dissimilarity in the high-field region (0.5 to 1.3 ppm) of the spectra which further indicate that the product from *M. leprae* contains a phenolic, esterified, and methoxylated glycol closely related but not identical to the mycoside A from *M. kansasii*.

The most salient and important difference in the NMR spectra of the glycolipids from the two sources was in the mid-field region, 4.0 to 5.5 ppm, i.e., the region of anomeric and ester group protons. The multiplet centered at about 4.80 ppm in both spectra was probably due to two protons of the ester link (Fig. 3A). However, other signals in the mid-field region differed. To distinguish 1-proton anomeric signals from others, both glycolipids were hydrolyzed with alkali followed by acid, and the NMR spectra were compared with those in Fig. 3. The ester proton signals had disappeared, but so also had those at 4.10, 5.03, and 5.43 ppm for the *M. kansasii* product and those at 4.24, ca. 4.42, and 5.45 ppm for the *M. leprae* glycolipid. Clearly, signals attributable to anomeric protons differed in the two spectra. The origin of the common signal at 5.12 ppm is not yet known; it is stable to both alkali and acid treatment.

To further examine the differences in anomeric proton signals, the sugars in the two glycolipids were compared by GLC of the alditol acetates (Fig. 4). Both contained three sugars. Those in the product from *M. kansasii* were recognized as 2,4-di-*O*-methylrhamnose, 2-*O*-methylfucose, and 2-*O*-methylrhamnose by GLC-mass spectroscopy and by cochromatography with the derivatized standard sugars. Thereby, the assignments already given to the inherent sugars of mycoside A (12) were confirmed. Combined GLC-mass spectroscopy readily identified the three sugars in the glycolipid from *M. leprae* as a 2,3-di-*O*-methyl-6-deoxyhexose, a 3-*O*-methyl-6-deoxyhexose, and a 3,6-di-*O*-methylhexose (Fig. 5). Demethylation of the sugar mixture and GLC of the alditol acetates, exactly as described previously (7), showed that the parent sugars were rhamnose and glucose. Hence, the three sugars in the *M. leprae* glycolipid are 2,3-di-*O*-methylrhamnose, 3-*O*-methylrhamnose, and 3,6-di-*O*-methylglucose.

Evidence for extracellular phenolic glycolipid in *M. leprae*-infected cells. The *M.*

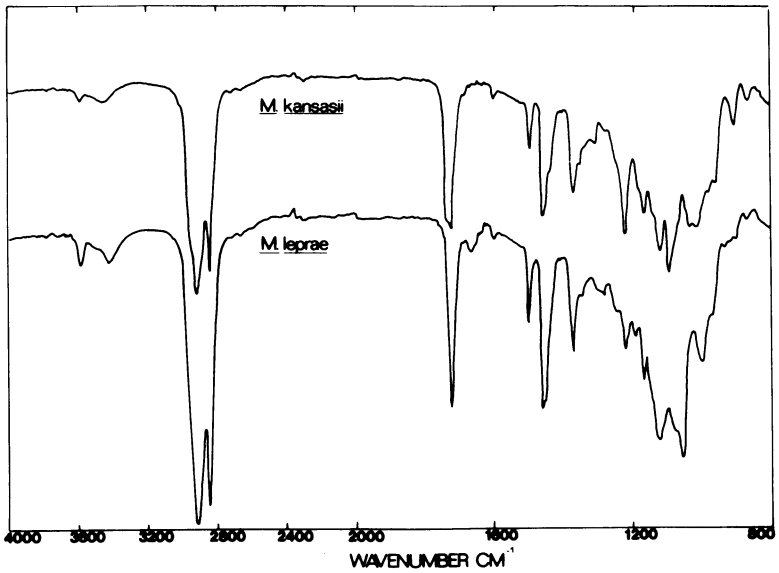

FIG. 2. *Infrared spectra of the phenolic glycolipids from M. kansasii and M. leprae. Measured as solutions, at a concentration of 25 mg/ml of CCl_4.*

leprae-free and detergent-free supernatant fluid obtained from the first centrifugation of homogenized infected liver proved to be a very rich source of the specific glycolipid (Table 1). Separation of this glycolipid from the plethora of host armadillo lipids was readily accomplished on account of its extreme apolarity; it was eluted from silicic acid with ether, whereas chloroform was required for removal of the earliest of the host lipids. Of the 2.17 mg of glycolipid per g of infected liver, 1.9 mg was not intimately associated with isolated *M. leprae*, although, as expected, there was a much higher specific activity in the organism than in infected liver (Table 1).

DISCUSSION

It is now clear that the outer surface of the majority of mycobacteria is characterized by a variety of rather exotic glycolipid antigens. To date, we have recognized two distinct classes. The best characterized of these are the C-mycosidic glycopeptidolipid antigens, present in all serovars of the *Mycobacterium avium-M. intracellulare-M. scrofulaceum* (MAIS) serocomplex (6, 7) and which, in the form of a minicapsule, confer smooth morphology on these bacteria and, perhaps, resistance to phagocytosis (2, 3). A second class, present in *M. kansasii*, *M. szulgai*, and others, consists of short oligosaccharides with fatty acyl and amino acyl attachments (P. J. Brennan, unpublished data). Both of these groups of glycolipid antigens are the basis of the famed Schaefer seroagglutination procedure (23, 24) for the identification and classification of atypical mycobacteria.

Present and past (5) evidence suggests a third class of mycobacterial glycolipid antigens, the phenolic glycolipids. The occurrence of a phenolic mycoside in *M. leprae* is not unexpected since lipids of this class have been found in other "typical" and "atypical" mycobacteria (9, 12, 21) and previous reports (5; Philip Draper, personal communication) had suggested such an event. The most interesting features of the present work are the unique sugar composition of the glycolipid and the fact that it is the major component of serologically active lipid preparations. However, we have not yet been able to unequivocally demonstrate that the pure glycolipid *per se* is serologically active; only impure preparations in the presence of "carrier" lipids are consistently active (5). Apparently, the extreme apolarity of the pure glycolipid interferes with its proper presentation in an aqueous serological assay. Other methods of presentation which should diminish the effects of the long hydrocarbon chains, such as incorporation of the glycolipid into liposomes (17), are now being considered. Also, as yet to be examined is the efficacy of the glycolipid in eliciting cellular immunity, the primary immunological response in cases of tuberculoid leprosy. It is possible that other lipids, present in small amounts, may be the antigenic principals; we have observed in *M. leprae* and in the liver milieu small quantities of more highly glycosylated phenolic lipids (S. W.

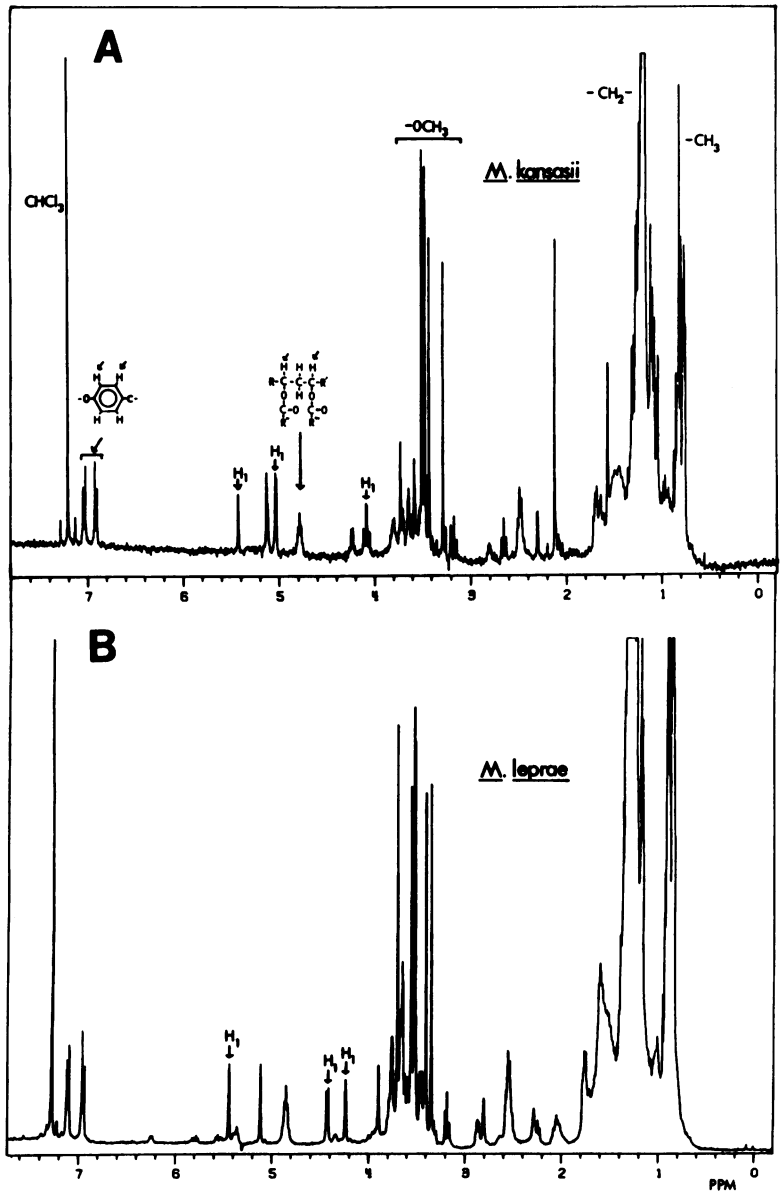

FIG. 3. *NMR spectra of the phenolic glycolipids from M. kansasii and M. leprae. Spectra were obtained at a concentration of 13.2 mg of lipids per ml of $CDCl_3$. Arrows indicate anomeric protons.*

Hunter, M. L. Goldhahn, and P. J. Brennan, unpublished data). Nevertheless, a comparison of the major phenolic glycolipid from *M. leprae* with the C-mycosidic glycolipids from the MAIS complex indicates that the former has all of the hallmarks of a species-specific antigen or hapten. For instance, the type-specific antigen of serotype (serovar) 2 (*M. avium* 2) of the MAIS complex also contains a unique 6-deoxyhexose-containing trisaccharide (7; P. J. Brennan, L. A. Hinton, and G. R. Gray, unpublished data). There is little doubt that the combination of sugars in the *M. leprae* product is unique. To our knowledge, the presence of 3,6-di-*O*-methylglucose in nature has not previously been reported, nor have we previously encountered 2,3-di-*O*-methylrhamnosyl and 3-*O*-methylrhamnose in one oligosaccharide. Therefore, although

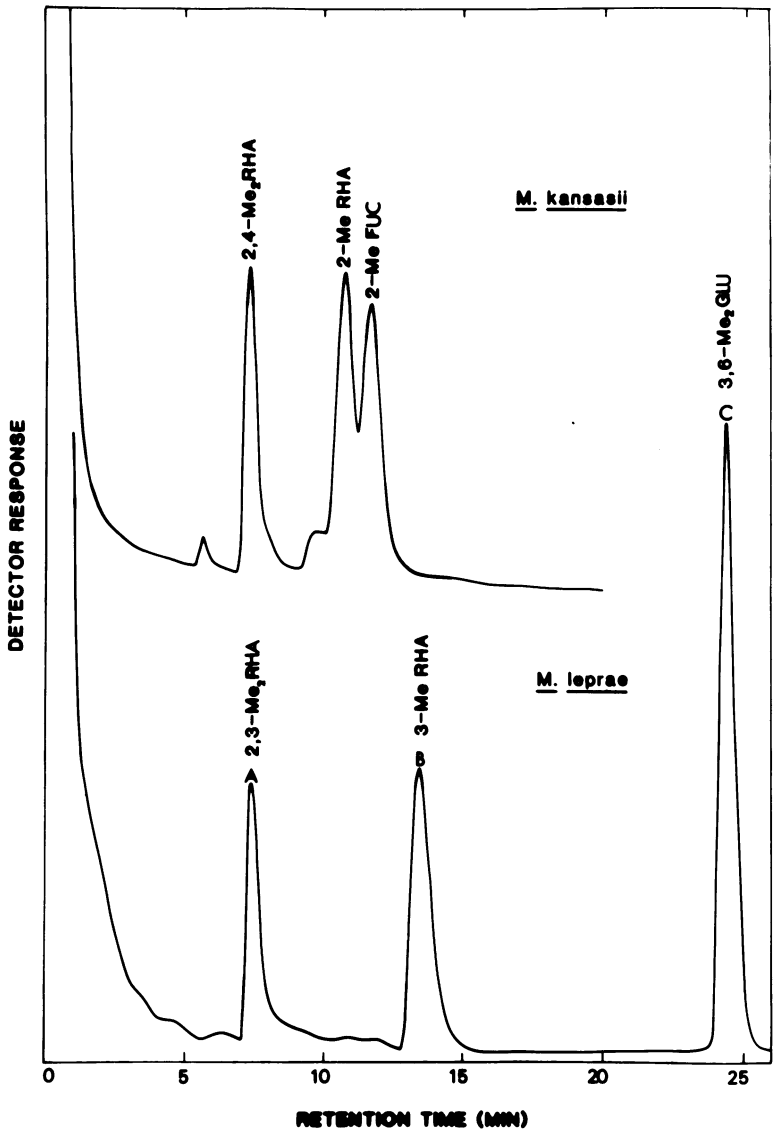

FIG. 4. *GLC of the alditol acetates derived from the phenolic glycolipids of M. kansasii and M. leprae. Purified lipids were hydrolyzed for 3 h at 100°C with 2 M CF_3COOH, reduced, and acetylated. GLC was conducted on a column (0.2 by 180 cm) of 3% SP-2340 on 100- to 120-mesh Supelcoport at 180°C for 18 min followed by a rise in temperature of 4°C/min for 10 min.*

the serological activity of the glycolipid is still a moot question, its unique thin-layer chromatographic mobility and sugar composition provide a chemical basis upon which *M. leprae* may be differentiated from all other mycobacteria.

Perhaps the most striking feature of this glycolipid is the quantity of it in the isolated bacillus (2% of the mass) and in the tissue environ (2.2 mg/g). We feel that it must be responsible for the so-called electron-transparent zone or "foam," an ill-defined structure of low density which surrounds the leprosy organism, particularly in tuberculoid tissue (14, 15). There was some indirect evidence based on staining properties (19) and solubility (11, 14) that the zone is lipid in nature; Ratledge (20) and, to some extent, Barksdale and Kim (2) have consistently referred to it as large and lipoidal. If this zone is in fact composed of the phenolic glycolipid, the inherent inertness of its structure would provide

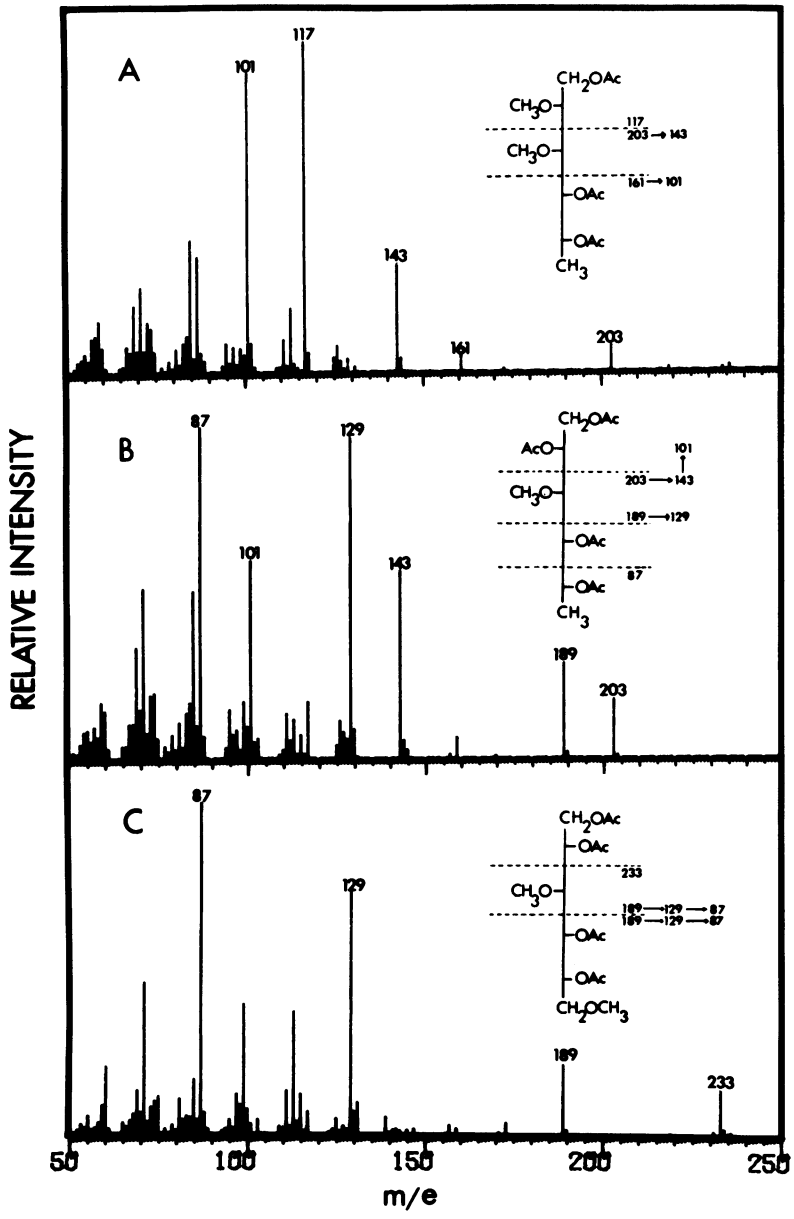

FIG. 5. *Mass spectra of the alditol acetates derived from the phenolic glycolipid of M. leprae (peaks A, B, and C; Fig. 4). Separation of alditol acetates was performed on a stainless steel column of 3% SP-2340 at 190°C isothermally for 8 min and programmed at 190 to 250°C at 6°C/min. Ion energy was 70 eV.*

an ideal antidote to the action of lysosomal enzymes.

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We wish to thank Leanne Goldhahn for her dedicated and skillful help.

TABLE 1. *Quantitation of phenolic glycolipid in M. leprae, M. leprae-infected armadillo liver, and M. leprae-free liver milieu*[a]

Source	Dry wt of source (g)[b]	Phenolic glycolipid recovered (mg)	
		Total	Amt per g of source
Liver	2.771	6.0	2.17
Liver supernatant fluid, *M. leprae*-free	2.627	5.0	1.90
M. leprae	0.048	1.1	22.92

[a] Irradiated infected armadillo liver was homogenized and centrifuged (10,000 × *g*; 10 min). The supernatant fluid, which was devoid of AFB, was extracted with $CHCl_3$-CH_3OH, and the glycolipid was purified by silicic acid chromatography. The 10,000 × *g* pellet was fractionated to yield pure *M. leprae* from which the glycolipid was also isolated. A separate portion of infected liver was homogenized and extracted to determine its content of glycolipid.

[b] From 10 g of wet liver; ca. 9×10^{10} AFB/g.

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